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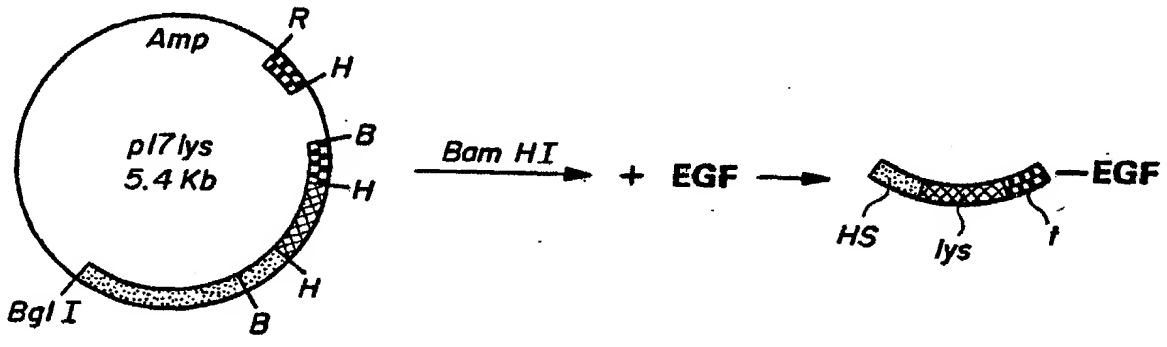

(57) Abstract

A piece of useful nucleic acid is linked to a cell homing or targeting factor, for instance a growth factor, a viral antigen or a factor capable of penetrating the cellular envelope, and the resulting coupled system is used to transfect and/or transform suitably selected receptor cells. This technique provides internalization in high yields, which results in gene expression or gene expression inhibition using anti-sense RNA techniques and constitutes a significant advance over classic transfection, e.g. with Ca^{++} ions. This new approach of targeting genes opens a new field for *in-vivo* applications of genetics.

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A METHOD OF MODIFYING THE METABOLISM OF EUKARYOTIC CELLS UPON INCORPORATION THEREIN OF FOREIGN SEQUENCES OF NUCLEIC ACIDS BY MEANS OF AN INTERNALIZING VECTOR

Field of the invention

The present invention concerns the introduction into eukaryotic cells of foreign nucleic acids capable to perform an active function in these cells, for instance enhance or inhibit some operations of the cell machinery, i.e. modify the cell metabolism.

For this, the invention proposes a suitable vector or vehicle capable of carrying said nucleic acids toward said recipient cells and promoting their penetration therein across the cell membrane without substantially impairing or inhibiting their inherent biofunctionality. Once incorporated in the cells, the foreign nucleic acids demonstrate said activity by accomplishing therein some specific function (or functions) like gene expression or repression, blocking viral activity, inhibiting cell proliferation or the like.

Hence, using the present vector, the invention also discloses a method for cell transfection i.e. for introducing and internalizing foreign sequences of nucleic acids into suitable eukaryotic recipient or host-cells, said nucleic acids being functionally active in said cells, i.e they are capable of altering the normal operation of the cell machinery. For instance, the foreign nucleic acids may promote the expression of new products in the cells, they may modify the cell metabolism or they may inhibit its growth or uncontrolled proliferation. In the cases when these nucleic acids are transported into the cell nucleus and become part of its genome, the results may account for cell transformation.

The prior art

Until now cell transfection (and/or transformation) has been carried out by several methods including, for instance,

working in the presence of Ca^{++} ions which promote the preferential uptake by cells of Ca-precipitated nucleic acids (F.L. GRAHAM et al. 1973, Virology 52, 456-467). Other methods also include for instance the direct injection of genes into oocyte nuclei (E.G. DIAKUMAKAS, Methods In Cell Biology, 1973 Academic Press, Vol. 7, 287-311) as well as the use of viral vectors (D.H. Hamer et al. 1979, Cell 18, 1299), or liposomes dispersions for carrying the genes into the cells (R. Fraley 1980, J. Biol. Chem. 255, 10431). Transfections can also be brought about with DEAE-Dextran (J.H. McCuthan, 1968, G. Natl., Cancer Inst. 41, 351) and by genetic transfer in high voltage electric fields (E. Neumann et al. 1982, Embo J. Vol. 1, No. 7, 841-845). See also R. Kucherlapati, Crit. Rev. Biochem. 1984, 16, 349-379.

Although all the above techniques have merit, they are not universally applicable. For example, with the DEAE-Dextran method exogenous DNA sequences are not efficiently integrated, this being in contrast to the method using calcium salts in which internalization of the DNA into the host genome frequently occurs. Direct injection into cells is tedious and requires highly skilled operators. Using a virus as a vector offers high yield transfections, but problems may arise from the simultaneous introduction of undesirable and possibly dangerous viral factors.

Furthermore, no transfection method exists by now which is applicable in-vivo for therapeutic purposes, e.g. in which foreign sequences of active nucleic acids can be applied to living organism to cure some deficiencies of the cell metabolism. Such possibility has however been mentioned by S.Y. Cheng, G.T. Merlino and I.H. Pastan, Nucleic Acid Research 11 (1983), 654-669 by saying "one of the medical applications of gene splicing technology could be the introduction of DNA into cells with genetic defects". It should therefore be clear that the term "nucleic acids" includes in the present invention DNA, duplex or single stranded RNA, anti-sense RNA, polynucleotides, oligo-nucleotides, nucleotide analogs, single nucleotides in general and all functional derivatives thereof.

There was therefore a need for improved transfection

techniques providing high internalization yields under mild conditions and of a highly selective character, i.e making it possible to selectively transfect and, in some cases, transform one particular species of cells in the presence of other cells of different kinds. This technique is especially attractive when the nucleic acids to be incorporated can genetically modify only specifically defined target cells, or, in the case of cancer therapy, internally promote the expression of a cytotoxic substance while leaving normal cells intact. Other applications include the internalization of active oligo and polynucleotide, e.g. anti-sense RNA which can hybridize to mRNA in the cell and thus inhibit cellular or viral functionality.

Summary of the invention

The method disclosed in annexed claim 1 is a very significant step toward the aforementioned desirable objectives.

In brief, a nucleic acid transporting vector or vehicle is synthesized in which the sequence or sequences of interest are coupled to a ligand which is capable to carry said sequences and bring it into contact with the recipient cells; then the ligand will promote or facilitate the internalization of the nucleic acid sequences in the cell and, in some cases, transfer said nucleic acids into the nucleus. After being internalized the foreign nucleic acids will display intended inherent bioactivity in the host-cells with consecutive expected changes in said host-cell's chemistry.

The transporting vector used in this invention can therefore be schematized as follows:

$$(HT) - B - (NT) \qquad (I)$$

in which (HT) designates the foregoing ligand (the head element) and (NT), the tail element, is the sequences of nucleic acids to be transported (DNA, RNA, poly-, oligonucleotides, etc.). B designates an intermediate element which connects (HT) and (NT). This can be a bridging or linking element. In

some cases B may be completely lacking, a chemical grouping of (HT) being directly linked (covalently or otherwise) to some bridging function of (NT), i.e. a 5'-phosphate or 3' or 5'-hydroxy group. The important feature of B besides connecting (HT) and (NT) is that it should not impede the biofunctionality of (NT) in the cell. One preferable way to achieve this task is to provide the vector with at least one bond which is easily cleaved by cellular enzymes, e.g. lysozyme enzymes. It should be noted at this stage that the construction of a system involving a gene coupled to a protein having high affinity for a cell receptor has been already reported (see S.Y. CHENG et al., Nucleic Acid Research 11 (1983), p. 654-669). However, although it was said, in the reference that such a system (the Chloramphenicol-acetyltransferase (CAT) gene bound to α2-macroglobulin) was internalized in 3T3-4 cells, no evidence was provided that the internalized DNA was capable of performing a function in the host-cells. In this case, the type of linker used to link the gene and the "arrow-head" was perhaps disactivating.

The "head" (HT) can also be named a "homing" or a "targeting" factor. The term of "homing" is used to define a factor which promotes or facilitates the penetration of the foreign nucleotides through the cell membrane but which has no or little specificity regarding cell recognition. Examples of homing factors are for instance low density lipoproteins (LDL), the "B" moiety of toxins such as ricin or abrin, some viral antigens, etc. Other examples of homing factors will be discussed in more detail hereafter.

The term "targeting factor" is preferentially used when, in addition to its internalizing properties, the "head" (HT) also has specific cell markers recognition properties, i.e. the (HT) factor will act as an "arrow-head" to direct the vector toward some specifically selected cells, for instance tumoral cells, while leaving other kinds of cells untouched, for instance healthy cells. Markers are defined as particular antigens or receptor sites possessed by a target cell, e.g. growth factor receptors like epidermal or platelet-derived growth factor receptors or receptors to immunoglobulins (see

EP-A-112.720) or α2-macroglobulin (see the foregoing reference of Cheng, Merlino and Pastan). Further examples of targeting factors will be provided later in the course of this description.

Component (NT) is a nucleotide or a nucleotide sequence which can be RNA, DNA or oligonucleotides complementary to specific RNA produced in the host-cell. Furthermore, the nucleotide sequences may be chemically modified in some cases to confer special properties thereto, e.g. to prolong the half-life thereof in the receptor cells or to be given duplex cutting activity. Alternatively, (NT) can designate a gene, e.g. recombinant DNA engineered to express products of interest in the host-cells. These products which can be transcriptional products (e.g. anti-sense RNA) or translational products will be detailed hereafter.

Components (HT) and (NT) may be connected together directly in some cases; in some other cases, they are linked together by an element (B) which can simply serve as a connecting element or, better, as a carrier for instance when many individual (NT) are transported together with only one homing or targeting factor (HT). This will also be detailed afterwards.

Another very important function of the linking element B is to allow connecting (HT) and (NT) without mutual functional inhibition, i.e. (HT) should promote the internalization and transport of (NT) to the recipient cells and not subsequently impede its biofunctionality in the cell. Reciprocally, (NT) should not prevent (HT) from achieving its transporting, targeting and internalizing function. Therefore the role of B, i.e. coupling (HT) and (NT) at appropriate distances and using appropriate binding sites, e.g. $-NH_2$, -SH, -OH or -COOH sites on the protein and 3'-OH, 5'-OH or 5'-phosphate on the nucleic acids, is important. This will be detailed later.

After being internalized, the nucleic acid sequences may accumulate in the cytoplasm or (depending on the nature of the (HT) element) they may be transported to the nucleus and effect cell transformation. Hence, the choice of the nucleic acids (NT) and that of the carrying factor (HT) will essen-

tially depend on the desired end-function of the polynucleotide, i.e. whether it should act directly in the cytoplasm (e.g. as blocking agent) or become part of the genome of the cell (e.g. a synthetic gene) and be expressed into useful products. In this connection, it should be remarked that useful products can include products expressed in-vitro in cultivated cells as well as products (e.g. RNA or proteins) produced in-vivo, for instance in living organisms, to modify the host-cells behaviour, e.g. to cure a genetic disease or to inhibit the growth of malignant cells.

Hence, whatever the desired objective and end-use of the foreign nucleotide internalized in the recipient cells, the present invention includes allowing or inducing the biofunctional activity of the nucleic acids in the host-cells until the effect resulting from this activity is achieved. Examples of such active functions will be discussed hereafter.

Detailed description of the invention

In the vector schematically respresented by formula (I) above, component (HT) preferably designates a protein capable of penetrating into cells either by receptor mediated endocytosis or through clathrin coated pits with subsequent accumulation in uncoated vesicles like viruses such as adenovirus, vesicular stomatitis virus, Rous sarcoma virus and Semliki forest virus. Thus, viral proteins used as the (HT) component can be specific or unspecific depending on the cells to be targeted and the nature of the viral factor. In this invention the use of viral extracts or specific viral antigens as the leader component is possible.

Proteins having specific affinity for receptors on the recipient cells are used as targeting factors (HT) in receptor mediated endocytosis. Suitable factors include epidermal growth factor, platelet-derived growth factors, urogastrone and analogues thereof, thyrotrypsin releasing hormone (TRH), nerve-growth factor (NGF) vaccinia virus growth factor and other specific viral factors, e.g. a viral antigen of the HIV virus (P.J. Maddon et al., 1986, Cell 47, 333) specific to the

T4-receptor typical of T4 lymphocytes but which can also be found on other cells.

In addition, other factors can be used as the (HT) component of vector (I), e.g. IgG, α-2 macroglobulin, somatomedin C, thiodothyronine, thrombine and the B-chain of many toxins including ricin, abrin, diphtheria toxin, etc.

The selection of the linker (B) between the (HT) and (NT) elements of vector (I) is important so as not to disable the functionality of both elements. Furthermore the chemical binding function of the linker should be selected in function to the available binding groups on both the leader protein and the attached nucleic acids.

With a targeting factor, like for example EGF, with a terminal arginine NH_2 group, binding can be effected directly by the method of C.F. CHU et al., 1983, Nucleic Acid Research 11, 6313-6529. This method involves activation of the 5'-phosphate groups of the polynucleotide chain with imidazole (IZ) at pH 6 in the presence of a suitable carbodiimide compound and subsequent displacement of this leaving group at pH 8,5 by the terminal aminogroup of EGF (EGF is actually a polypeptide containing 53 amino-acids, see Savage et al., (1972) J. Biol. Chem. 247, 7609). This is schematically illustrated in Fig. 1.

Although it is noted that CHU et al. suggested that constructs carried out according to the above scheme might be used to achieve the objects of the present invention, they did not report the actual existence of DNA-protein adducts and the effective use of such adducts to permanently modify the operation of recipient cells.

The scheme of the above coupling method is:

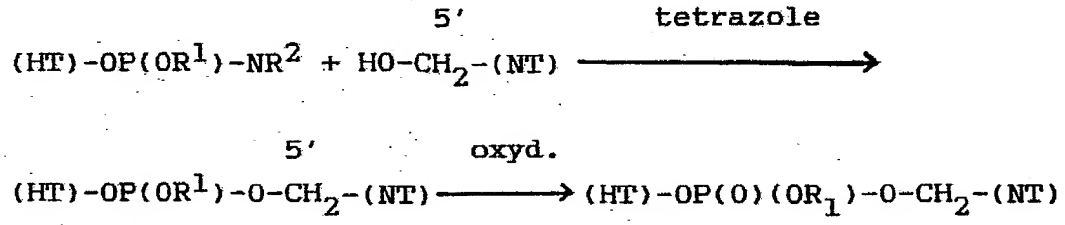
$$(NT)\text{-}OPO_3^{-2} + IZ \longrightarrow (NT)\text{-}OP(O_2^-)\text{-}IZ$$

$$EGF\text{-}NH_2 + (NT)\text{-}OP(O_2^-)\text{-}IZ \rightarrow EGF\text{-}NH\text{-}(PO_2)\text{-}O(NT)$$

In the above scheme, tetrazole can be used in place of imidazole.

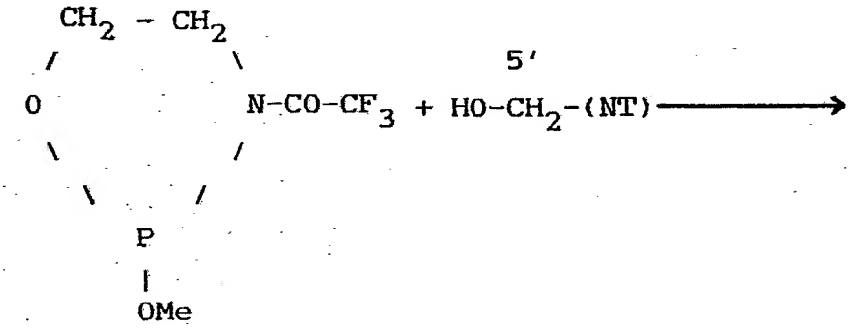
Another direct coupling is illustrated by the reaction of the 5'-OH of the sugar moiety of the nucleoside with a termi-

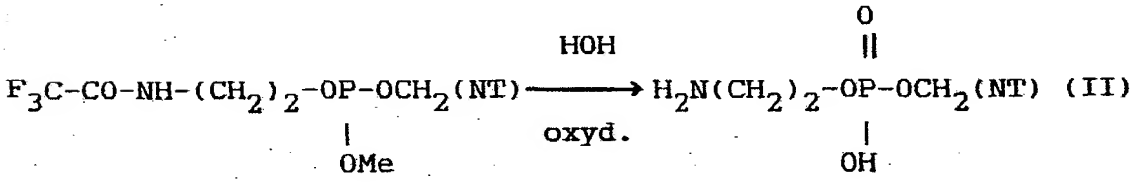
nal phosphoramidite group attached to the (HT) component. This scheme, which is illustrated below, is similar to that used for the synthesis of oligonucleotides by the known step-by-step processes and is convenient for binding to proteins having available -OH or -SH groups for coupling (see BIOFUTUR, No 50, Oct. 1986, supplement p. 1-18)



In the above scheme R^1 and R^2 are lower alkyl, e.g. methyl or isopropyl.

Another very useful route for coupling (NT) is to provide it with an amino-alkylene link which can be thereafter bound to (HT) by usual means, e.g. use a cyclic phosphinamidite released by Applied Biosystems (see Applied Biosystem User Bulletin, issue No 38, November 3, 1986). This is illustrated below





The "aminolinked" nucleic acid (II) can thereafter be coupled

to a primary amino-group of (HT) by usual means, for instance by the glutaraldehyde bridging reaction (see S. Avrameas (1969) Immunochemistry 6, 43).

In order to procure a linker (B) (see formula I) which can be easily cleaved by cellular enzyme after internalization, it may be advantageous to insert between (HT) and (NT) one or more peptide bonds provided by suitable amino-acids. Examples of oligopeptides suitable for the present purpose have been disclosed in co-pending PCT application WO 87/03891, which is incorporated here by reference, and defined as $-(CO-CHR-NH-)_m-$ (III) in which R can be hydrogen (glycin), methyl (alanine), benzyl (phenylalanine) and the like, and m can be an integer from 1 to about 10 or greater. The method for inserting such an oligopeptide between a protein and, say, compound II can follow a number a routes according to well known techniques. According to one example between many, an oligopeptide is first synthesized according to usual means, e.g. protecting the aminogroup of a first amino-acid with a benzocarbonyl or tert.butoxycarbonyl, coupling a second amino-acid in active ester form in the presence of dicyclohexyl-carbodiimide and hydroxybenzotriazole, removing the amino protective group by hydrogenation or by hydrolysis, repeating the coupling with a third amino-acid (with protected amino group) and repeating the procedure until all amino-acid units have been added.

Then the activated ester of the oligopeptide is reacted with compound II to provide an amide link, the amino group at the other end is deprotected and coupled with the protein by using a conventional protein coupling reagent, for instance glutaraldehyde as mentioned before.

The above scheme is schematized as follows (Y designates the activating ester radical).

$$\text{tert. Bu-OCO-(NH-CHR-CO)}_m\text{-OY} + \text{II} \xrightarrow[\text{deprotection}]{\text{amidation}}$$

$$H\text{-}(NH\text{-}CHR\text{-}CO)_m\text{-}NH(CH_2)_2\text{-}OP(O)(OH)O\text{-}(NT) \xrightarrow[\text{linker}]{(HT)}$$

$$(HT)\text{-}NH(CH_2)_5\text{-}(NHCHRCO)_m\text{-}NH(CH_2)_2\text{-}OP(O)(OH)\text{-}O\text{-}(NT)$$

It is evident that the oligopeptide can also be inserted in the reverse position, i.e. the activated ester can be reacted with the protein and the remaining $-NH_2$ coupled with the amino group of II using the same or equivalent techniques.

Many linking agents to conjugate the end functions of a peptide chain with proteins have been disclosed in the prior art. For instance, EP-A-112.720 (Y. KATO et al.) discloses attaching the following groups to the terminals of peptides: (i) $HS(CH_2)_2\text{-}CO\text{-}$, (ii) $HS(CH_2)_2\text{-}CH(NHAc)\text{-}CO\text{-}$, (iii) $HS\text{-}CH(AcOH)\text{-}CO\text{-}$ and the following groups to a protein (IgG): (a) -CO-phenylene-N-maleinimide; (b) $-CO\text{-}(CH_2)_2\text{-}SS\text{-}2\text{-}pyridine$:

These are applicable in the present invention; coupling for instance (i) with (b) in the present invention provides an adduct with a dithio-bond:

$$HT\text{-}CO\text{-}(CH_2)_2\text{-}SS\text{-}(CH_2)_2\text{-}(CO\text{-}CHR\text{-}NH)_m.....NH(CH_2)_2\text{-}OPO_3\text{-}NT$$

Furthermore, attaching a group of formula O_2N(2-carboxy-p-phenylene)$\text{-}SS\text{-}(CH_2)_2\text{-}CO\text{-}$ to an oligopeptide is useful to bind to an -SH function of a protein, thus providing an $-SS(CH_2)_2-$ between the protein and the polyaminoacid.

The group B in adduct (I) can also act as a carrier of more than one sequence of nucleic acids. For instance B can be a polypeptide bearing side groups for attaching a plurality of nucleic acid sequences (10, 50, 100 or more) to be carried to the recipient cells. Suitable polypeptides are for instance polyglutamic or polyaspartic acid.

Adducts of comparable structure but in which the attached groups are not nucleic acids but cytotoxic organic molecules have been disclosed in EP-A-112.720 and also in copending US application 07/82,244 filed August 6, 1987 incorporated herein

as reference.

Basically, the intermediate group B in this embodiment can be represented by formula:

$$-(CO\text{-}CH\text{-}NH)_n\text{-}H \quad \text{with side chain on CH:} \quad (CH_2)_p\text{-}CO\text{-}(NH\text{-}CHR\text{-}CO)_m\text{-}A\text{-} \qquad (IV)$$

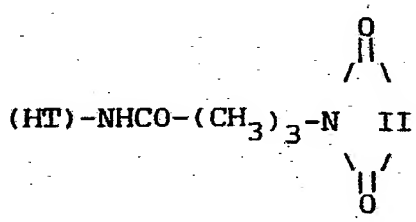
in which p = 1 or 2, m is zero to about 10, n is 20 to 300, and A is a linker between the oligopeptide $(NH\text{-}CHR\text{-}CO)_m$ and the nucleic acid NT, for instance the chain $-NH(CH_2)_2$-phosphate disclosed hereabove. A structure of this kind is particularly useful for delivering a plurality of oligonucleotides, e.g. relatively short chains of anti-sense DNA or RNA into cells using only one homing or targeting factor. The efficiency of such vector is hence strongly amplified as compared to a vector containing only one nucleic acid chain per leading head. The efficiency of such a vector can be further enhanced if the oligonucleotide comprises, in addition to sequences which block cellular or viral RNA, sequences which prolong the useful life of the internalized oligonucleotide.

The coupling of a carrier such as schematized by formula IV with a homing or targeting protein is done as disclosed in the case of linking compound III. A related technique is fully developed in the foregoing US application 07/82,244 and related EP-A-87.201.490.7 application. This technique is based on first making a thiolated polyglutamic or polyaspartic active ester of formula:

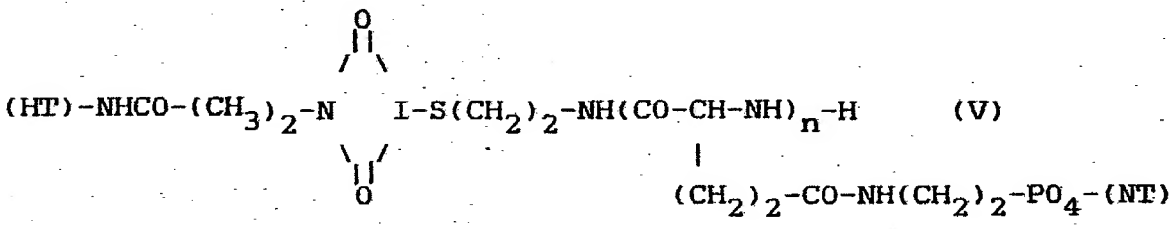
$$HS(CH_2)_2\text{-}NH\text{-}(CO\text{-}CH\text{-}NH)_n\text{-}H \quad \text{with side chain on CH:} \quad (CH_2)_2\text{-}COOY$$

Then reacting the activated side ester chains with the amino derivatized nucleotide compound II and finally reacting the intermediate adduct of the carrier and the nucleotide with a maleinimido derivative of the protein, i.e. a compound of

formula:



The coupling occurs through addition of the sulfur atom to the maleinamido double bond to provide the following compound:



Compound V is an embodiment of compound IV in which m = 0. Variants can be achieved in which m ≠ o. In such cases the side arms bearing the nucleic acids comprise oligopeptides the size and the type of which can control the enzymatic release in the cell of the nucleic acids transported therein.

In should be noted that many other coupling agents can be used in the synthesis of the structures usable in this invention. Some are disclosed in PIERCE Bioresearch Products Technical bulletin, 1983, Volume V, Revised 84-87 available for instance from Kontron Instruments AG, CH-1052 Lausanne.

Having now briefly described the main embodiments of this invention, specific embodiments will now be described in more detail.

In a first embodiment, a selected piece of DNA or RNA, for instance a gene of interest was placed under the control of adequate promotor and terminator sequences; it was then coupled to an appropriate cell homing or targeting vector vehicle selected not to impede the ultimate functioning of the DNA or RNA in the cell. When the resulting system was put in the presence of the target cells, the vector-DNA complex did penetrate into said cells, whereby the DNA or RNA of interest

was internalized in high yields and became functional therein, for instance, it was subsequently expressed. Homing factor means here as said before that although the ligand promotes or facilitates internalization, i.e. it provides a route to the interior of the cell membrane, it does not necessarily recognize a particular type of cells by means of specific receptors of these cells. In contrast, a targeting factor will additionally provide such recognition and direct the adduct toward specific kinds of cells identified by markers.

Actually, the construction of a system involving a gene coupled to a protein having affinity for a cell receptor has been already reported (see. S.Y. CHENG et al., Nucleic Acid Research 11 (1983), p. 654-669). However, although it was said that such a system (the Chloramphenicol-acetyltransferase (CAT) gene bound to α2-macroglobulin) was internalized in 3T3-4 cells, no evidence was provided that the internalized DNA was capable of performing a function in the host-cells. In this case, the type of linker used to link the gene and the "arrow-head" was perhaps disactivating.

Naturally, the present method is not limited to transfection procedures using only one piece of nucleic acid; co-transfection with two or more sequences is also possible using one or several different homing factors, this being done depending on the case, simultaneously in one step or, successively, in several steps. When effecting a co-transfection with two or more different polynucleotides, for instance, fragments a and b, these fragments can be introduced by linking them separately to the target vectors. Alternatively a and b can be linked together on a same vector unit, the selection of one particular technique depending on the desired results and the operational conditions. Also, if suitable linkers are used between the ligand and the polynucleotide, e.g. a polyfunctional amino-acid such as polyglutamic acid or polylysine (see EP-A-87/201.490.7), a large number (10 - 100 or more) of polynucleotide sequences can be attached to one homing or targeting factor.

In one experimental model developed to demonstrate the feasibility of the concept, i.e. cell transformation, the

foreign nucleic acids included sequences for regulating the cell development, e.g. using certain eukaryotic cells such as the NIH/3T3 line, said additional sequence was an oncogene of cell transformation visible in tissue culture and in-vivo cellular origin which promoted after inoculation into warm-blooded animals such as nude mice which consecutively develop tumors.

It should be noted that this technique is particularly useful when the foreign gene is designed to express functional products of commercial interest and, consequently, multiplication of the modified host-cells can be performed on a large scale (for instance, industrial production of protein expressed by recombinant DNA dominated by stress inducible promotors).

In the case of in-vivo applications (e.g. treatment of tumors), the foreign nucleic acid can be a heat inducible gene, e.g. a gene coding for a cytotoxic protein drug and driven by heat-shock controlling sequences. In this case the targeting vector is selected for its ability to recognize the malignant cells and promote internalization exclusively therein; then, after effective in-vivo transformation of the tumor cells, heat is applied locally whereby the gene is expressed and the malignant cells are inhibited or killed while leaving the normal cells intact. When cell recognition is not essential, e.g. in the case of fighting some viral diseases, the homing factor can be a factor promoting cell internalization, such as viral antigens, low density lipoproteins, part B of ricin or abrin, etc.

Brief description of the figures

Fig. 1 is a schematic illustration of the coupling of an oligonucleotide to a cell targeting agent (the epidermal growth factor, EGF is employed here as an example).

Fig. 2 is a diagram illustrating the construction of a selected piece of DNA (HS-lys-t/EGF), i.e. a chicken lysosyme gene driven by a human heat-shock promotor and terminated by the SV40 terminator sequence; and the coupling of this selec-

ted DNA to EGF.

Fig. 3 is a photograph of an electropherogram providing evidence of specific RNA formation under stress in A-431 carcinoma host-cells transfected with the HS-lys-t/EGF coupled system.

Figs 4a-4c are photographs of cell cultures providing evidence of transformation of "normal" NIH/3T3 cell lines into tumor cells after internalization and expression of an oncogene transported by the EGF homing factor. The black dots represent the "foci" of transformed NIH/3T3 cells.

Fig. 5a is a photograph illustrating the formation of tumors in nude mide 3 weeks after inoculation of oncogenically transformed NIH/3T3 cells ("foci") represented in Fig. 4. Fig. 5b shows a control nude mouse three weeks after inoculation with NIH/3T3 cells treated with EGF only.

Fig. 6a represents, under magnification, a culture of a human squamous carcinoma cell line A-431 showing the presence by specific staining of the cell membrane, of EGF receptors. Fig. 6b represents, under the same magnification, control cells (WI-38 human embryonic fibroblasts).

First embodiment

In this first embodiment of the present invention, the epidermal growth factor (EGF) was linked to a 700 bp DNA restriction fragment from the oncogene Ha-ras labelled by NICK Translating with ^{32}P dCTP and the resulting complex (I) (see Fig. 1) was used to transfect cultures of A-431 cells; the use of these cells (see Fig. 6a) was convenient as they carry a large concentration of EGF receptors (see M.D. Waterfield (1982), J. Cell. Biochem. 20, 149-161).

This coupling was effected according to B.C.F. CHU et al., (1983), Nucleic Acid Research 11 (18), 6513-6529. This method which has been described earlier, in this application is schematically illustrated in Fig. 1.

It has already been noted that CHU et al. speculated that constructs carried out according to the above scheme might be used to achieve the objects of the present invention, however, they did not report the actual existence of DNA-protein

adducts and the effective use of such adducts to permanently modify the operation of recipient cells.

After 3 hours of incubation in the instant case, the degree of internalization of the radioactively tagged DNA was measured. It was found that 45% thereof was in the cell cytoplasm, and nucleus, with none in the cellular membrane. A control experiment was performed identically, but in the presence of a 5 fold excess of free EGF for competition. This time the amount of radioactive label in the cytoplasm and nucleus DNA was reduced to 16% providing evidence that internalization of the complex did proceed through the EGF receptors.

Naturally, this embodiment is not limited to using the 5'-phosphate as linker. Many other linkers of the kind disclosed herebefore which do not inhibit or impede the desired biofunctional capacity of the incorporated polynucleotide are also possible.

Second embodiment

In a second embodiment, EGF was bound to a piece of DNA schematized by the formula HS-lys-t in which HS is the human heat-schock promotor sequence (Dreano M. et al. (1987) Gene 49, 1-8), lys is the chicken lysozyme gene and t is the SV40 terminator. The scheme illustrating the preparation of this adduct (compound II) is represented in Fig. 2.

Compound II was introduced into the culture medium of monolayer cultures of A-431 squamous carcimona cells and, after a period of incubation, the cultures were subjected to a heat stress (4 hours at 42°C), after which the production of lysozyme-specific RNA was ascertained by extraction followed by S-1 hybridization. The results appear in the electropherogram of Fig. 3.

In fig. 3, lane 1 is the test lane. The lysozyme RNA appears as the dark band (A). Lane 2 (control) is obtained from a culture transfected by the method of the prior art (in the presence of calcium phosphate). Lane 3 illustrates a control experiment in which no compound II was added. Lane 4

is for calibration purposes using markers.

These results conclusively demontrate the validity of this technique of internalizing DNA in functional form, i.e. in a form which can be inducibly expressed. Naturally, this technique is not limited to structural genes placed under inducible expression, but can be applied to constitutively expressed genes as well.

Third embodiment

In a third embodiment the ras oncogene was coupled to EGF; the resulting complex (III) was added to cultures of NIH/3T3 cells and left overnight. After two weeks, the formation of many "foci" was noted, providing evidence of stable cell transformation (i.e. stable incorporation of the "ras" DNA into the "normal" genome). "Foci" are agglomerates of densely packed cells with a refringent phenotype and a tendancy to pile up. The "foci" are the black dots on the photographs of the Petri dishes respresented in Fig. 4a. Fig. 4b illustrates the results of a control experiment in which transfection was carried out with the oncogene (without targeting agent) in the presence of calcium salts according to the prior art. Fig. 4c illustrates another control experiment in which only free EGF for adding to the NIH/3T3 cells was used. No observable "foci" were produced this time. The "foci" induced tumor formation when injected into nude mice. Thus, the tansformed colonies ("foci") were picked and grown in-vitro until reaching 5×10^6 cells. Then these cells were injected into athymic nude mice. As a control, 5×10^6 NIH/3T3 cells treated with EGF (100 µg/ml), were also injected into nude mice. Three weeks after, all the mice inoculated with cells grown from the "foci" developed tumors (Fig. 5a), in contrast to the control mice which did not (Fig. 5b).

In a second series of controls, injection in nude mice of cells grown from "foci" of NIH/3T3 cells, transfected with the ras oncogene using the calcium chloride method, also induced tumors as expected.

This demonstrates further that the oncogene, introduced

into cells according to the invention, is expressed and acts in a stable fashion.

Fourth embodiment

A fourth embodiment here concerns the homing or targeting of anti-sense RNA (DNA) or of genes engineered to express anti-sense RNA after internalization by a viral antigen.

More specifically, a nucleotide sequence [called here anti-sense RNA (DNA)], which can be RNA, DNA or modified oligonucleotides, complementary to a specific RNA produced in a specific cell population, is targeted and internalized by a viral antigen. Alternatively, a gene construct expressing anti-sense RNA is targeted and internalized by a viral antigen. These compounds are designed to specifically inhibit viral infection by blocking the expression of certain viral proteins. Anti-sense RNA has been shown to inhibit mRNA translation through intra-cellular hybridization. The anti-sense RNA (DNA)-viral antigen complexes of this invention are designed to be used in-vitro as well as in-vivo. As an additional interest to this embodiment is the fact that whole genes can be targeted and internalized with viral antigens, providing a way of bringing new protein synthesis capabilities to cells in the form of DNA.

Viruses exert their action on specific cell types. It has been described that the action of viruses is mediated by the specific targeting and internalization of the whole virus particle. This specificity and the internalization are determined partly by viral antigens. These viral proteins also allow the virus to escape the lysozomal degradative pathway by fusion with the endosomal membranes at a ph of 5 to 6. For instance, the specificity of the HIV virus (LAV or HTLVIII) for the receptor of the T4 lymphocyte is determined by one of its viral antigens (see M. March (1984), Biochem. J. 218, 1; P.J. Maddon et al., (1986), Cell 47, 333). In this invention, such viral antigens can be used to target and internalize oligonucleotides and genes.

Many viruses , such as adenovirus, vesicular stomatitis

virus, Rous sarcoma virus and Semliki forest virus, enter cells by sliding horizontaly on the cell membrane to clathrin coated pits and subsequently accumulate in uncoated vesicles. They enter the cells using the same pathway as many growth hormones, such as epidermal growth factor, or platelet-derived growth factor, or molecules such as alpha 2-macroglobulin, somatomedin C, thiodothyronine, thrombine, shigella and diphtheria toxins (A. Helenius et al., (1980), J. Cell Biol. 84, 40; S. Dales (1973), Bacteriol Rev. 37, 103; R.B. Dickson et al. (1981) J. Cell Biol. 89, 29; M.C. Willingham et al. (1981) International Cell Biology 1980-1981, Ed. H.G. Schweiger, Springer Verlag, Berlin, pp. 613-621, I. Pastan et al. (1981), Science 214, 504. Targeting oligonucleotides with a viral antigen offers new possibilities over using antibodies and hormones because the route followed to penetrate a specific cell is the same as the one used by the virus. Driving anti-sense RNA (DNA) with a viral antigen makes the model even more elaborate and allows one to develop new targeted therapeutic drugs which should be without substantial side effects.

The internalized viruses are sequestered in receptosomes which fuse to lysozomes. Although the lysozomes bring a large cocktail of proteolytic enzymes, the viruses manage to escape in the cytoplasm and replicate. The escape of material from the lysozomes is seen in many other cases; for instance some DNA escapes from lysozomes in all transfection procedures. It has been shown for instance (see R. Kucherlapati et al., (1984), Crit. Rev. Biochem. 16, 349) that whatever the mode of transfection, e.g. electroshock, $CaCl_2$ or DEAE-dextran, the internalized DNA is always exposed to lysozomal enzymes. Furthermore, although in a different area, many cytotoxic drugs designed as endocellular drug releasing systems enter the cells and are degraded in the lysozomes, releasing then the drug, which goes to the cytoplasm or to the nucleus (see R. Arnon et al. (1983), Targeted Drugs, Ed., E.P. Goldberg, Wiley Public, p. 23). The same patheway is usable with vectors carrying genes or anti-sense RNA and transported by viral antigens.

Anti-sense RNA is known to inhibit endocellular protein

synthesis.

Several papers have been published describing protein synthesis inhibition using anti-sense RNA. For example R.Y.L. To et al., (1986), Molec. Cell Biology 6 (12), 4758 have described the inhibition of retroviral replication using a virus expressing anti-sense RNA. I.J. McGarry and S. Lindquist, I.J. McGarry et al. (1986), PNAS 83, 399 (USA) have inhibited the synthesis of a heat-shock protein using a heat inducible construct expressing anti-sense RNA. J. Hunt et al. (1986, FEBS 4082, 206 (2), 319 have described the inhibition of the EGF receptor synthesis using anti-sense RNA vectors transfected in a squamous carcinoma cell line. The use of anti-sense RNA as a way of inhibiting virus infection has been often suggested as a novel approach J.G. Izant et al. (1985), Science 299, 345; E.C.M. Mariman (1985), Nature (letter), 318, 414; R. Tellier et al., (1985), Nature (letter), 318, 141. A potential limitation of the method is the amount of the anti-sense molecules needed for the inhibiting of viral infection.

In the present invention, the viral antigen (or a portion of which) is extracted and purified from whole virus, or it can be synthesized by classical peptide synthesis; then it is coupled to DNA (or RNA) by the aforementioned methods. The oligonucleotides can contain modified nucleotides designed to prolong half life or to give special properties such as cutting RNA strands when it is hybridized to them. The oligonucleotide-antigen coupling can be done using a variety of methods such as described by CHU et al. (1983), Nucl. Ac. Res. 11, (18), 6513.

The following operations are then performed to demonstrate the viability of the embodiment: the vector is added to cells infected by a virus, whereby:

the viral antigen recognizes its specific receptor;
the complex viral antigen-anti-sense RNA (DNA) enters the cell;
the anti-sense RNA (DNA) escapes the receptosome;
the anti-sense RNA (DNA) hybridizes specifically to the newly synthesized viral RNA;

the viral infection is inhibited.

Alternatively, in the case the viral antigen is used as a vector to target and internalize a gene, the protein expressed by this gene can be an antiviral agent or serve another purpose not directly connected with the viral infection.

Fifth embodiment

A fifth embodiment here (which is to be paired with the foregoing third embodiment) concerns the construction of a plasmid expressing an inverted segment (e.g. the KpnI-XmaI fragment) of the ras gene. Alternatively, different inverted fragments of the ras gene can be used as well. These fragments may be synthesized by oligonucleotide synthesis, isolated from genes or otherwise.

The role of the ras oncogene has been widely described (T.Y. Shih and M.O. Weeks, 1984, Cancer Investigation , 2 (2), 109-223; C.J. Marshall, 1986, J. Cell. Sci. Suppl. 4, 417-430). It has been shown to transform in-vitro cell lines as well as primary cells. These cells become morphologically different and are tumorigenic in nude mice. The ras encoded protein is located in the cell membrane and is analogous to a subunit of G proteins (H.R. Bourne and K.A. Sullivan, 1986, Cancer Surveys, 5 (2), 257-431). The G proteins are second messengers in growth factor induced signals. The ras homologous subunit of the G proteins has a GTP binding property which regulates the intensity of the signalling. The ras encoded proteins only have a low binding affinity for GTP, transmitting therefore a constitutive signal to the cell (see the above Bourne reference). It is postulated that this mechanism transforms the cell.

It appears that the normal ras protein has a role in the signalling pathway of growth factors (see above Bourne reference). Studies on the sequence of this normal ras protein and the transforming ras protein have shown that single point mutations can be responsible for the transforming activity of the latter. These point mutations are located on the 12th,

13th, 59th, 61th and 63rd amino acid of the protein P21 (see above Bourne reference). In a different series of studies it has been demonstrated that the overexpression of the ras gene can transform cells in culture and that the injection of antibodies against the ras oncogene in the cell do not replicate their DNA after serum stimulation. This demonstrates that a precise dosage of the ras protein is necessary so that the cell does not progress to malignancy. Therefore, controlling the normal and/or the mutated ras expresion seems to be a key feature for tumor inhibition.

Using anti-sense RNA technology, the technique disclosed in this invention permits to design a system in which anti-sense RNA is expressed so as to hybridize in situ specifically to the mutated portions of the cellular (or viral) encoded transforming ras RNA. Alternatively in a manner similar to that described in the fourth embodiment, oligonucleotides which can hybridize to the transforming ras RNA can be introduced into the cells.

The steps involved in the first approach are:

1. Construct a plasmid expressing anti-sense RNA in mammalian cells. The ras fragment is the inverted KpnI/Xma I fragment of ras comporting the 12th amino acid point mutation or a different inverted fragment of the ras gene under control of a constitutive or inducible promotor. These fragments can be synthesized by oligonucleotide synthesis or extracted from a cloned oncogene.
2. Attach the linearized plasmid to a vector according to the foregoing techniques.
3. Apply the conjugates to various mammalian cells in culture.
4. Mesure the endogenous ras expression by S1 mapping or by immunocytochemistry.
5. Evaluate tumor regression by injecting the conjugates in nude mice carrying tumors (e.g. NIH/3T3 cells transformed by ras)

An example of such a construction is schematized below:

1st approach: EGF- [promotor inducible or constitutive | inverted gene fragment e.g. KpnI/XmaI fragment of ras | terminator]

(ref: (C. J. Tabin et al. (1982), Nature 300, 143-152)

2nd approach: EGF —o (o = anti-sense oligonucleotide)

EGF—o o o o o o (o = anti-sense oligonucleotide)

Note: the oligonucleotides are attached by degradable linkers (stable in blood and degradable in the cell).

EGF— Carrier (e.g. polyglutamic acid)

X X X X }
Y Y Y Y } Linker (amino acids X,Y,Z)
Z Z Z Z }
0 0 0 0 Oligonucleotide (0)

The second approach is summarized as follows:

1. Synthesize oligonucleotides hybridizing to the mutated portions of the transforming ras RNA.
2. Couple the oligonucleotides to the carrier (preferably that defined by formula (IV) above and couple to the targeting factor (HT), for instance EGF
3. Transfect the construction into cells.
4. Evaluate the cell internalization of these conjugates.
5. Measure the effect of these conjugates on the ras expression in cells bearing EGF receptors (S1, immunocytochemistry).
6. Evaluate tumors repression by injecting the conjugates in nude mice carrying tumors (e.g. the NIH/3T3 cells transformed by ras).

In this approach small oligonucleotides complementary to a defined portion of RNA can be ligated or synthesized such as to get a repeated sequence. This sequence is targeted so that a single homing agent can inhibit a plurality of distinct RNA

molecules by using, as a carrier, polyglutamic acid whose free carboxylic chains are used as δ-linkers for the oligonucleotides. The construction is designed to be plasma stable but degradable in the targeted cells.

This adduct is designed, a) to circumvent the steric problems of using a sequence of repeated oligonucleotides built to hybridize to many RNA molecules, and b) to allow an intracellular release of a large amount of anti-sense oligonucleotides. In addition these oligonucleotides can be provided with a terminal amino acid which will protect them from DNA degrading enzmymes.

The first object of this procedure is to produce a targeted drug against ras transformed cells. There are a variety of tumors in which ras has been suspected to play an active transforming action. We have here a new DNA/Protein conjugate with internalizing properties targeted to these cells. EGF is preferably used as a targeting vector. However, other specific or non-specific targeting and homing devices (such as growth factors, monoclonal antibodies, Fab fractions, LDL, transferrin, viral and tumor specific antigens) can be used as the driving units.

These homing devices can be attached to different anti-sense RNA expressing constructs, or to oligonucleotides or to polynucleotides, so that these constructs can be applied for the development of genetic medicaments against several diseases such as viral diseases, cancer or genetic disturbances.

Sixth embodiment

A sixth possible embodiment concerns the use as targeting factor, within the scope of this invention, of an antigen from HIV (the AIDS causing virus). (HT) involved in this process seems to be the gp 41 protein. A short stretch of about 29 amino acids of this antigen has been demonstrated to bind selectively to the T4 receptor of lymphocytes, the main target of this virus. This stretch is schematized as:

DTITLPCRIKQIINMWQEVGKAMYAPPIS (international amino acid code)

This amino acid sequence can be used to target polynucleotides as well as antiviral agents such as AZT, acyclovir, HPA-23 or suramin.

This stretch of 29 amino-acids is synthesized by usual means; then it is coupled as a targeting factor (HT) to a carrier (for instance that shown in formula IV) of viral anti-sense RNA. The vector is to be used for treating AIDS patients. This is an example of using a (HT) factor to target antiviral agents in the form of oligonucleotide analogs.

Seventh embodiment

A seventh embodiment deals with the treatment of some forms of cancerous diseases caused by the overexpression of some oncogenes. Therefore this embodiment is related to the foregoing fifth embodiment and the techniques disclosed therein are similarly applicable.

Cells can be transformed by the overexpression of some oncogenes. For example evidence has merged that the neu oncogene is overexpressed in a large amount of breast tumors. The fact that the overproduced neu oncoprotein has a transforming activity has been elegantly demonstrated using antibodies to revert the phenotype of neu-transformed NIH/3T3 cells. Sequence homology has been demonstrated between neu, c-erb-B and the epidermal growth factor receptor (EGFR). Anti-sense RNA protein inhibition therefore provides good possibilities to revert the action of such oncoproteins. Morover, one single oligonucleotide may by sufficient to inhibit all three proteins. The approach described here is to fix anti-sense oligonucleotides to a polymer targeted by the epidermal growth factor. This construct, a targeted anti-sense oligonucleotide releasing system, is designed to have 1) plasma stability, 2) specificity properties, 3) internalizing properties, 4) lysosomal biodegradability properties and 5) antitumor activity. The fact that a large amount of oligonucleotides can be delivered by one EGF molecule adds value to this model.

Several proto-oncogenes, including the EGF receptor gene, neu, c-erb-B, ras and myc have been found to be amplified in human tumors. The presence of amplified copies of the N-myc oncogene in human neuroblastomas and of the neu oncogene in breast tumors have been correlated with aggresiveness of the tumors. The EGF receptor, c-erb-B-2 and the neu oncoprotein have been shown to have a close sequence similarity. These oncoproteins are good models for antisense RNA inhibition experiments. Anti-sense RNA expression constructs or synthetic anti-sense oligonucleotides efficient against these proteins have a high pharmaceutical potential value if the efficient in-vivo administration routes proposed in this disclosure are used.

EGFR is found in large quantities in squamous carcinomas from the bladder, breast, lung, stomach, skin and the brain. (J.R.C. Sainsburg et al., 1987, The Lancet, June 20, 1398-1402; Patent cytotoxic drug EP-A-87.201.490.7). The association of enhanced expression with more agressive tumor behaviour has been reported for these tumors. A plasmid expressing anti-sense RNA against the EGF receptor has been constructed and been shown to reduce the EGF receptor concentration.

c-erb-B-2 (first isolated in a human mammary carcinoma) is amplified in human renal, gastric and salivary gland adenocarcinomas. It has been found in 58 of 257 human adenocarcinoma examined and amplification was also shown in 26% of 189 human breast cancers. Amplification of the c-erb-B-2 gene was shown to correlate with overexpression (D.J. Venter et al., 1987, The Lancet, July, 69-71). In the breast cancer study amplification correlated with poor prognosis and was a stronger predictor of disease progression than the presence of oestrogen or progesterone receptors. Recently it has been demonstrated that overexpression of this oncogene is necessary for NIH/3T3 transformation (P.P. Di Fiore et al., 1987, Science, 237, 178-182).

Neu (first isolated in a rat ethylnitrosourea induced neuroblastoma) is strongly transcribed in neuroblastomas and glioblastomas. Several lines of evidence shown that neu is an activated form of the erb-B-2 gene. It has been demonstrated

to be amplified in many cases of breast cancer. It transforms NIH/3T3 cells, rendering them tumorigenic in nude mice. Antibodies to the oncogenic rat neu protein was shown to inhibit tumor formation in-vivo (J.A. Drebin et al., 1985, Cell, 41, 695-706).

Hence, the reduction of the expression of these proteins should revert the malignancy of some tumors. Targeted endocellular anti-sense oligonucleotide releasing systems to these proteins is a valuable approach. Constructs consisting of an EGF molecule attached to a endocellular degradable polymer carrying several oligonucleotides within the meaning of the present invention are valuable anti-cancer medicaments.

Homologous amino acid sequences can be found in the extracellular part and the intracytoplasmic part of the proteins expressed by EGFR, c-erb-B2 and neu genes (C.J. Bargmann et al., 1986, Nature, 319, 226-234). These homologous amino acid sequences have some homologous nucleotide sequences which can serve as target for anti-sense RNA. This provides a good opportunity to use a same oligonucleotide to inhibit the synthesis of three different cancer-related genes. These three proteins of interest have external and internal domains. The best homology between these oncoproteins is found in a tyrosine kinase segment and in a short segment of the external domain. There is a 9 amino acid stretch in the extracellular sequence that is homologous in all three proteins. It contains an 8 nucleotide sequence homologous in all three proteins, an 11 nucleotide sequence homologous in c-erb-B-2 and EGFR, an 11 nucleotide sequence homologous in EGFR and neu and a 15 nucleotide sequence homologous in c-erb-B-2 and neu. In the intracytoplasmic sequence, there is a 28 amino acid stretch that is homologous in all three proteins. It is in the domain coding for the tyrosin kinase part of the protein. In this region long stretches of homologous nucleotide sequences can be found for all three oncoproteins. These sequences can be used to inhibit the protein synthesis of the three oncoproteins. Of course, conjugates using sequences that are not homologous to all three genes can be used. The literature references backing this embodiment include the following: C.I.

Bargmann et al., Nature, 1986 319, 226-230, P.P. Di Fiori et al., Science, 1987, 237, 178-182, J.A. Drebin et al., Cell, 1985, 41, 695-706, J. Hunts et al., FEBS 4082, 1986, 206 (2), 319-322, G.T. Merlino et al., Mol. cell. Biol., 1985, 5 (7), 1722-1734, J.R.C. Sainsbury et al., The Lancet, June 20, 1987, 1398-1402, D.J. Slamon et al., Science, 1987, 235, 177-182, D.J. Venture et al., The Lancet, 11 July, 1987, 69-71, T. Yamamoto et al., Nature 1987, 319, 230-234.

The experimental approach here is quite similar to that of the previous embodiments. Briefly, oligonucleotides are fixed directly to EGF or to an EGF-targeted polyglutamic acid to generate a targeted anti-sense endocellular oligonucleotide releasing system. The latter provides the possibility of internalizing many oligonucleotides for one EGF molecule. Alternative experiments include direct targeting of anti-sense RNA expressing constructs by EGF. In the case of EGF, the oligonucleotide is linked to a polymer-EGF construct and used to down regulate the EGF receptor of A431 cells. Then, the following tests are made to check the effect of the vector:

a) Peroxydase-labelled immunotests to show EGF receptor concentration decrease,
b) ^{125}I-labelled EGF to show EGF affinity diminution,
c) ^{35}S-methionine to show cell division reduction,
d) In-vivo tumorigenicity of the pretreated cells.
e) Inhibition by in-vivo treatment with the targeted oligonucleotide by observing the reduction of tumor progression.

This is an example in which a ligand may inhibit its own attachment. This provides a new form of antagonist. In the case of the neu gene, the oligonucleotides are linked to a polymer-EGF construct as above and used to inhibit the oncogenic synthesis in NIH/3T3 cells transformed by neu. This is followed by the tests below:

a) demonstrate the disappearance of the transformed refringent phenotype of neu transformed NIH/3T3 cells.
b) test growth of these NIH/3T3 cells in soft agar.
c) test tumorigenicity of these cells in nude mice.
d) treat nude mice carrying neu transformed NIH/3T3 tumors

with anti-sense oligonucleotides linked to EGF.

In the case of c-erb-B2, the oligonucleotides are linked to EGF as usual and used to inhibit cell transformation in neu transformed NIH/3T3 cells. The laboratory work is the same as for neu but using c-erb-B-2 transformed cells. Cross experiments with c-erb-B-2 transformed cells or with cells with high levels of EGF are useful.

Of course, as in the other embodiments of this invention, the use of EGF as a targeting and internalizing factor can be extended to other molecules having similar properties (other growth factors, monoclonal antibodies, Fab fractions, LDL, transferrin, viral antigens, etc..). The transporter can also be different to polyglutamic acid (homo- or co-polymers of amino acids with or without side chains, dextrans, etc...). The oligonucleotides can be, or not, modified to prolong their half-life. In addition, the model can be extended to the targeting of "polyoligonucleotides" with or without biodegradable bonds between each oligonucelotide.

Eighth embodiment

Another eighth embodiment is now described in which the invention may be applied to the inhibition of poliovirus infection using anti-sense RNA.

It is known that viruses carry unique genetic material. Therefore, specific viral sequences can easily be distinguished from cellular sequences. Anti-sense RNA is a very specific tool which can block viral infection. It has been used to inhibit the replication of HIV, the AIDS virus (P.C. Zamecnik et al., (1986), PNAS 83, 4143). Thus, for instance, the genetic material of poliovirus is composed of a positive sense single-stranded RNA. The infectious cycle of this virus can be blocked with anti-sense RNA (or oligonucleotides) complementary to the viral RNA. This inhibition can occur both at the replication or at the translation level.

Plasmids and oligonucleotides are targeted and internalized using for example EGF as a vector. Oligonucleotides are delivered in large quantities by targeting endocellular oligo-

nucleotide releasing systems. These systems as well as targeted genes can also be used in-vivo and are described here as new antiviral agents. Poliovirus serves to demonstrate the usefulness of this accomplishment for other applications to different viruses including HIV/Human Immunodeficiency virus), Herpes, Influenza, Rhinovirus, Cytomegalovirus, HTLV (Human T-cell leukaemia virus).

The vectors described here are designed to be used as antiviral agents in therapy and, for some of them, in prevention. Poliovirus is used as a model to demonstrate the concept. Two routes are proposed for inhibiting poliovirus infection:

I) Targeted gene constructs expressing anti-sense RNA.

II) Targeted anti-sense oligonucleotides or anti-sense oligonucleotide releasing systems.

I) Gene constructs expressing anti-sense RNA can be constructed and provided with a homing or targeting unit (HT). For example, a plasmid contains a neomycin resistance gene under control of the SV40 promotor, the SV40 replication origin, the heat-shock promotor controlling the anti-sense RNA synthesis (i.e. the inverted polio-sequences) and an ampicillin resistance gene (pl7poneo).

Using EGF as the driver (HT), the vector can be tested by experiments involving:

1) Establishment of neomycin resistant cell lines (Wish, Hela, Cos) with targeted pl7poneo or, alternatively, transfect and amplify pl7poneo in Cos cells. These cells, when heat shocked, express large amounts of anti-sense RNAs.

2) Infection with different viral dilutions of these cell lines before or after heat treatment. The inhibition of poliovirus infection can be evaluated by:
   - trypan blue counts
   - soft agar infected colony counts
   - ^{3}H-thymidine incorporation

3) Control experiments include:
   - testing for the presence of hybrids (viral RNA/anti-sense RNA) by treating cytoplasmic extracts with

RNAse H and hybridizing the remaining duplexes with a nick translated probe

- testing for the presence of viral proteins using ^{35}S-methionine incorporation.
- repeating all tests with untargeted p17poneo using standard transfecting procedures.

II) Two limitations to the use of synthetic anti-sense oligonucleotides are their half-lives in the cell and the concentration of molecules that can be introduced into cells. The advantage of using synthetic oligonucleotides is that one can modify their chemistry to prolong their half-lives. Introduction of these oligonucleotides by the present EGF-targeted vector method, e.g. EGF-targeted polymers carrying large amounts of oligonucleotides can remedy the above drawbacks.

The practical work involved here includes:

1) The synthesis by usual methods (gene machine) of the oligonucleotides complementary to the viral RNA. Some of them are attached directly to EGF and some of them are attached to a targeted carrier i.e. targeted polyglutamic acid. This carrier is stable in the blood stream and degradable in the targeted cell compartment.
2) Cells are allowed to react with these free (or targeted) oligonucleotides before or after being infected with poliovirus.
3) The effect is evaluated by:
   - trypan blue counts
   - soft agar infected colony counts
   - ^{3}H-thymidine incorporation
4) Control experiments include:
   - treating cytoplasmic extracts with S1 nuclease, spotting the genetic material and revealing the S1 protected fragments with a ^{32}p-probe,
   - using ^{32}p-anti-sense oligonucleotide to inhibit protein synthesis, S1 digesting and running on a gel,
   - testing for the presence of viral proteins using

^{35}S-methionine incorporation.

The targeted genes, the targeted anti-sense RNA-expressing gene constructs, the targeted anti-sense RNA and the targeted anti-sense oligonucleotide releasing system can be used in-vivo. They can be injected in the blood stream, targeted to specific cell populations and internalized in these cells.

This is related to the targeted drug releasing systems disclosed in same aforementioned references which have been shown to be effective in animals. Therefore, in addition to the concept of gene targeting, one of the main innovations here is that the targeting, the cell internalisation and the releasing effect which has been used for drugs (EP-A-87.201.490.7) have been extended to nucleic acids. By analogy, this combination should be as effective as the targeted drugs systems but with the remarquable additional advantage to have limited undesirable side-effects. Such compounds are therefore powerful antiviral agents and some embodiments will be used extensively in cancer therapy.

The gene constructs and the targeted compounds described in this embodiment illustrate the feasibility of using genetic constructs involving different promotors and sequences, different targeting moieties (other growth factors, monoclonal antibodies or their Fab fractions, LDL, transferrin, viral antigens, etc...) as well as a variety of polymers (homo- or co-polymers of amino acids with or without side chains, dextrans, etc...).

The described achievement opens a new field for the development of completely new pharmaceutical compounds for human and animal health care. In addition, based on the same concepts, new antiviral agents can be conceived for plant applications.

Ninth embodiment

A ninth embodiment of this invention concerns the use of a toxin moiety as a different homing factor in the vector of formula (I) for transporting nucleic acids into recipient

cells. This toxin moiety, can be part B of certain toxins which recognize cell receptors and normally promote the entry of the second moiety A, which is toxically active.

Indeed, certain toxins such as ricin and abrin are composed of two parts, A and B. The A part is the toxic moiety whereas the B part recognizes cell receptors and promotes the entry of A. Once separated chemically, A and B fractions are totally devoid of toxic activity.

This embodiment deals with the attachment of nucleic acids to purified B chains to promote their entry into the cell. This system permits the introduction of nucleic acids into eukaryotic cells in-vitro and in-vivo. Conjugates of B-chains with anti-sense oligonucleotides constitute a very interesting application of this approach.

This toxic A fraction has been used to construct immunotoxins (see for instance I.S. Trowbridge et al., (1981), Nature, 294, 171-173; J.J. Bjorn et al., (1985), Cancer Res., 45, 1214-1221; E.B. Blythman et al., (1981), Nature, 290, 145-146; W.K. Miskimins et al., (1979), Biochem. Biophys. Res. Comm., 91 (1), 143-151). The toxins most widely utilized are diphtheria toxin, the exotoxin secreted by Corynebacterium diphtheriae, and the plant toxins abrin and ricin from the seeds of Abrus precatorius and Ricinius cummunis respectively. They all consist of two polypeptide chains, A and B, linked by a disulphide bond. The separated chains are virtually devoid of cytotoxic action. The B-chain contains binding sites which recognize molecules that are present on the surface of virtually all cell types. Abrin and ricin recognize galactose-terminating glycoproteins and glycolipids whereas the receptor for diphtheria toxin is probably a 150,000 MW glycoprotein. After binding via the B-chain, the toxin is taken into the cell by receptor-mediated endocytosis (see S. Olsnes et al., (1982), Toxic lectins and related proteins. In Molecular Action of Toxins and Viruses (ed. P. Cohen & S. van Heyningen), pp. 51-105. Elsevier Biomedical Press, New York). The A-chain then appears to traverse the membrane of the endocytic vesicle and enter the soluble phase of the cytoplasm. There, the A-chain catalytically inactivates the cell's machinery for

protein synthesis. In this embodiment like in previous ones, use is made of anti-sense RNA or oligonucleotides which are sequences complementary to a specific cellular or viral RNA. These sequences hybridize to the RNA and block the translation or, if they are chemically modified, cut the hybridized RNA. If the targeted sequence have enzymatic activity (such as some RNA that can cleave RNA), it may also specifically degrade the hybridized RNA. Experiments have demonstrated the validity of using anti-sense expression plasmids, anti-sense RNA or oligonucleotides (see for instance J.G. Izant et al., (1984), Cell, 36, 1007-1015. J.G. Izant et al., (1985), Science, 229, 345-352). Viral infection has been inhibited in-vitro but the extension of the experiments in living organisms is difficult since oligonucleotides do not freely permeate in cells. (C.H. Agris et al., (1986), Biochemistry, 25, 6268-6275, R.Y.L. To et al., (1986), Molecular and cellular biology, 6 (12), 4758-4762, P.J. Green et al., (1986), Ann. Rev. Biochem., 55, 569-597. T.J. McGarry et al., (1986), PNAS, USA, 83, 399-403, C.J. Marcus-Sekura et al., (1987), Nucleic Acid Research, 15 (14), 5749-5763).

One way of circumventing this problem is to attach these oligonucleotides to a molecule which will internalize into the cells.

Here, we develop the possibility of using the B fraction of toxins to internalize products such as nucleic acids. These conjugates are generally (although not always) unspecific due to the fact that the B chains recognize a large variety of cells. However, since anti-sense oligonucleotides are very specific they probably have no detrimental effect in environments where they are not functionally active.

A and B fractions generally linked by disulfide bonds can be separated by reducing the S-S bond. The two resulting fractions are separated by the methods disclosed in the aforementioned references and the -SH groups serve for the chemical coupling of nucleic acids endocellular nucleotide carrier vehicles as disclosed before, nucleotide analogs etc.

One route to bind the -SH group of a protein to a polyaminoacid carrier has been mentioned herebefore and is conve-

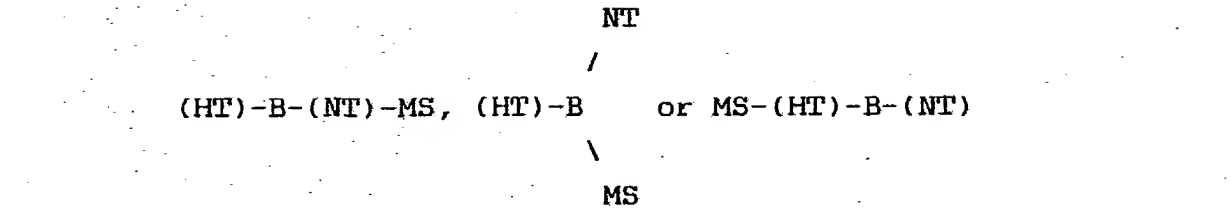
nient for this application.

As before, the vectors composed of a toxin B moiety as driving factor (HT), a middle linker or carrier portion and, bound thereto, nucleic acids of interest, can be used to transport said nucleic acids into receptor cells, e.g. in living organisms and across cell membranes. This tansporting system is particularly valuable for molecules that do not freely (or efficiently) permeate through the cell membrane because of their charge, size or chemical composition, for example. Examples include nucleic acids and nucleotide analogs. In the case of translation inhibitors such as anti-sense nucleic acids that bind to RNA, the specificity of the sequences may be good enough to avoid targeting.

The embodiment described here can be applied in disease prevention and in therapy, and forms the basis for the design of new drugs with anticancer, antiviral or antibacterial effects. Systems acting on gene expression can then be constructed, to provide a large variety of new medicaments including blood disease applications.

Problems of efficient gene transfer also exist in plants. Therefore, it may also be useful to use this kind of system to introduce nucleic acids in plants. This is a new approach and a different class of pesticides enabling to generate better breeds or even permitting intra- or inter-species reproduction. If targeting is necessary, the use of the previously described technology is also possible.

One interesting improvement to the vector of formula I which constitutes one of the key features of this invention is the addition thereto of an amino acid sequence capable of mediating the transport of the conjugate (or at least of the nucleic acid moiety) into the cell nucleus. Such amino acid sequences, called here migration signal (MS) have been found in viruses and have been disclosed by C. Wychowski et al., (1986), EMBO J. 5, 2569. One sequence is represented by the first eight NH_2-terminal amino acids of SV40 VP1 (Ala-Pro-Thr-Lys-Arg-Lys-Gly-Ser) using a (MS) sequence, the vector (I) can then be represented by the formulae:



(MS = migration signal; HT = Homing, Targeting; B = linker; NT = nucleic acid)

Preferably, this sequence is inserted at a site before the polynucleotide, e.g. at an end of intermediate bridging element (B) of formula (III) or in a corresponding position in the carrier element of formula (IV). Such an insertion will impart to vector (I) enhanced cell transforming properties.

The following examples illustrate the invention in more detail. It should be noted that the operational conditions disclosed have not been optimized yet. More refined conditions are expected to increase the yields and provide still improved results.

In other embodiments designed to demonstrate the operability of in-vivo gene therapy, targeted complexes composed of a gene coding for a cytotoxic protein (e.g. the ricin diphtheria or pseudomonas toxin gene) linked to a homing vector such as EGF or any other tumor cell specific marker can be injected into cancerous animals, either locally or in the blood stream. Then, if the promoter selected makes the gene heat inducible, local heating the subject induces the expression of the toxin only in the region of the tumor, whereby the latter is destroyed, without harmful effects to healthy tissues. Otherwise, similarly built targeted complexes but comprising other promotors, e.g. for controlling expression by chemical induction, can be used similarly.

E X A M P L E 1

Coupling of DNA to EGF

Double-stranded DNA was attached to EGF molecules according to the aforementioned B.C.F. CHU et al., reference (see also EP-A-86.810.347.4)

Genes were excised from plasmids by digestion with endonuclear restriction enzymes, leaving 5' terminal phosphates. 0,5 to 1 mg of digested plasmid was then run in a 1% agarose gel, and the band corresponding to the gene of interest cut and purified from the gel by electroelution as follows: the gel containing the DNA was introduced into a 5 ml plastic pipet at the end of which was placed siliconized glass fiber and dialysis bag. The pipet was dipped into TAE buffer (Tris-acetate 0,04M + EDTA 0,001M) and electrophoresed at 150 V for 2 hours. The current was then inversed for two minutes; the DNA recuperated from the dialysis bag, and precipitated twice with ethanol. The first time the DNA was dissolved in water, and the second time, in 20 µl of imidazole buffer 0,1M pH 6.0. The electroelution was controlled by running 1 µl of this solution on a 1% agarose gel.

Formation of a 5'-phosphorimidazolide on the DNA molecules was obtained by saturating the solution with N-ethyl-N'(3-dimethylaminopropyl-carbodiimidehydrochloride) (EDC) (FLUKA). The reaction was performed overnight at 4°C with agitation. Aminolysis of imidazolide of the DNA, by the amino terminal extremity of EGF was performed by buffering the solution with 3 volumes of 100 mM citrate buffer (C.B.) pH 8.5, and by adding 4 to 10 µg EGF (Sigma), and traces of EGF labelled with ^{125}I. (New England Nuclear). Leaving it for 48 hours at 4°C with agitation generated the EGF-DNA conjugate. The free EGF was then separated from the EGF-DNA complex by running the solution over G75 SEPHADEX (column 100 x 2 cm) using a 0,1M ammonium carbonate solution, pH 7.0. The first peak of radioactivity is attributed to the radiolabelled EGF-DNA conjugate. The corresponding fractions were pooled, distributed in aliquots and freeze-dried. Up to 85% of the DNA had an EGF molecule bound to it, as calculated from the DNA concentration (absorption at 260 mm) and the number of EGF molecules (amount of radioactivity bound to the DNA).

The EGF-DNA conjugate was kept lyophilyzed in plastic vials at 4°C until rehydration in growth medium for use.

EXAMPLE 2

Selection of test cell lines based on evidence of receptor concentration on the cell membrane using indirect immunoperoxydase staining

Indirect immunoperoxydase staining on A-431 squamous carcinoma and W-138 normal fibroblast human cell lines was performed on trypsinised cells in 35 mm PVC plates. The surface was pre-treated with phosphate buffered saline (PBS) pH 7.2, the excess was then removed and the PBS washed cells (10^5/well in 50 µl/PBS) were added to the plates and centrifuged for 5 minutes at 2000 rpm. 50 µl/well of 0,5% glutaraldehyde in cold PBS were then added to the dish and incubated for 15 minutes at room temperature. After two rounds of washes with PBS, the wells were filled with 100 mM glycine in a 0,1% BSA solution and allowed to rest for 30 minutes at room temperature to inhibit the excess of glutaraldehyde. After two PBS washes, indirect immunoperoxydase staining was performed by first denaturing the cells with an ice cold mixture of 99:1 ethanol-acetic acid for 30 minutes at 4°C. During this period, 3 µl of antibody were diluted in 1 ml PBS + 1% foetal calf serum (FCS). The wells were then washed twice with PBS and incubated for 5 minutes with a solution of 20% FCS in PBS. This was then replaced by 200 µl/well of the antibody solution, and incubated for 30 minutes at room temperature. The wells were than washed twice with PBS, once with PBS + Tween (0,1%) and once again with PBS. 200 µl/well of a 1/400 dilution of swine anti-mouse peroxydase conjugated antibody (DAKO) IN PBS-1% PVS was then added and incubated for 30 minutes at room temperature. The conjugate was then washed twice with PBS, once with PBS + 0,1% Tween and twice with distilled water. The in situ coloring was achieved by incubating the cells at room temperature with a solution of 10 ml 0,01M phosphate buffer pH 6.0, 5 µl 35% oxygenated water and 100 µl of 1% orthodianisidin (Merck) in methanol.

Fig. 6a represents A-431 tumor cells. The presence of large amounts of EGF receptors therein is indicated by the

dark staining. Fig. 6b shows the weakly stained W-138 cells containing much lower amounts of EGF receptors which were used as a control.

E X A M P L E 3

EGF ~ DNA internalization study

EGF was attached to DNA fragments as described in Example 1. The fragment used in this experiment is a 700 base pair probe labelled with αP^{32} ATP by nick-translation using the New England Nuclear System. The internalisation study protocol used here is adapted from a method used by Hillman, G.M. and Schlessinger, J. (Amer. Chem. Soc., 1982, 21, 1667-1672). Briefly, the cell monolayer is incubated with the EGF-DNA mixture, after which the receptors are extracted from the membrane and the cells solubilized for counting the internalized DNA.

Monolayer cell cultures in Petri dishes of 3 cm diameter, were incubated for 3 and a half hours at 37°C with the EGF-DNA complex, labelled with p^{32}, in solution A (4 parts DMEM, 1 part of 50mM Tris, 100mM NaCl and 0,1% BSA adjusted at pH 7.4). The cells were then washed four times with ice-cold PBS + (1mM $CaCl_2$ and 1 mM $MgCl_2$). A 50% trichloroacetic acid solution was added in a proportion of 1/5 to the poolded solution A and PBS + and counted on a scintillation counter. The cell membranes were destabilized by treatment on ice with 200 mM acetic acid and 150 mM NaCl (solution B) for 6 minutes. The solution B was then removed and the cells washed twice with solution B. These pooled solutions were counted for p^{32}. This treatment releases the EGF receptors bound to the cell surface. The cells were then completely dissolved in 0,2N NaOH. The radioactivity found then represented the internalized EGF-DNA complex.

To control whether the radiolabelled DNA entered the cell using the internalizing EGF receptor system, competition experiments were set up. The medium was saturated with EGF molecules, thus competitively preventing a large proportion of the

EGF-DNA complex from interacting with the EGF receptor. 2 µg of free EGF was added to the medium containing the radiolabelled EGF-DNA complex, and the internalized radioactivity was counted after 3 and a half hours of incubation at 37°C.

In these experiments, as much as 45% of the radiolabelled EGF-DNA complex added to the medium was found in the intracellular compartment after 3 and a half hours of incubation. Under the same conditions, when the medium was saturated with free unlabelled EGF, the internalized EGF-DNA complex was reduced to 1/3, displaying only 16% of internalized product.

These results taken together provide evidence that DNA can be carried into the cell using the EGF receptor system.

E X A M P L E 4

Gene construct conjugated to EGF enters the cell and is transiently expressed

A human heat inducible promotor, fused to a chicken lysozyme gene has been used as a model in a transient expression system. Such a construct, termed pl7lys, has been demonstrated to function in monkey COS cells and in xenopus oocytes (Dreano M. et al., 1986, Gene in press). pl7lys was condensed with EGF (as described in example 1) and the EGF - pl7lys complex was mixed in complete growth medium and added to A-431 monolayer cell cultures as follows.

The cells were first washed twice with DMEM without serum left to incubate for 4 hours in medium without serum. The EGF - pl7lys complex was then added to 1 ml of medium without serum. After 3 hours incubation at 37°C, 9 ml of DMEM, 10% FCS was added. After an overnight incubation, the cells were heat shocked at 42°C for 4 hours and lysed for extraction of the RNA.

RNA extraction was performed by treating PBS washed cells with a solution containing 10mM Tris pH 7.5, 1mM $MgCl_2$, 10mM NaCl, 1% SDS and 1 mg/ml fresh proteinase K. The cells were left in this solution on ice for 1 hour after which NaCl was added to make 0,3 M. The cell debris were scraped off and

centrifuged 10 minutes at 11000 r.p.m. in an airfuge at 4°C. The supernatant was then removed and precipitated in ethanol for 5 minutes in a dry ice-ethanol bath. After a 15 minute centrifugation, the supernatant was discarded and the pellet redissolved in 20 µl of 10 mM PIPES, pH 6.9, 0,4M NaCl, 1mM EDTA, 50% formamide. The hybridization with a 400 bp. fragment of the lysozyme gene construct was performed according to H.G. Stummenberg and M.L. Brinstiel (P.N.A.S., USA, 1982, Vol. 79, pp 6201-6204). Aliquots of about 30 µg of total RNA were used for each assay. The SI protection assay was carried out according to Weaver, R.F. and Weissmann, C., (1979, Nucl. Acids Res., 6, 1175-1193 except that DNA fragments were not heated in pure formamide but resuspended and denatured directly in hybridization buffer (Dierks et al., 1981, Cell, 32, 695-706). Nuclease SI was from PL/Pharmacia. After digestion, excess RNA was eliminated in 0,1N NaOH at 100°C for 3 min. The protected fragments were analysed on 7M urea, 8% acrylamide gels (0,4 mm). End-labelled HaeIII fragments of pBR322 were used as molecular weight markers.

As one can see on figure 3, the lysozyme gene has been internalized and expresses RNA. Therefore, it is shown here that an internalized gene stays functional. Of course, if the gene was cloned in an inverse orientation, the transcribed RNA would be anti-sense and could repress the translation of "sense" RNA encoded by a "sense" gene. Naturally, in this example, HT and the nucleic acid can be different. As an illustration of the possibility of inhibiting gene expression using anti-sense genes, we have, in another set of experiments, co-injected in Xenopus oocytes "sense" and "anti-sense" (gene reversed in Sal I site) CAT genes under control of the heat-shock promotor (see figure in example 7, p. 46). The latter having efficiently inhibited the CAT synthesis, we concluded that targeting "anti-sense" constructs was an evident application of "sense" gene targeting.

EXAMPLE 5

A gene construct linked to EGF enters the cell and brings about stable transformation

A well documented system is the transformation of NIH/3T3 mouse fibroblasts by ras oncogenes using calcium chloride transfection technique (Graham, F.L. and Van der Eb, A.J. , 1973, Virology, 52, 456-467 Wigler, M. et al., 1979, PNAS 76, 1373-1376). The transformed cells form foci of refringent cells that have lost contact inhibition and can grow in athymic mice.

We have fused the humas ras oncogene to an EGF molecule as described in example 1, and have tested this complex on NIH/3T3 cells. Confluent monolayers of NIH/3T3 cells were washed twice with DMEM without serum, and were incubated in this medium for 4 hours. After this, the EGF-ras complex was added to the medium and allowed to incubate for 3 hours at 37°C. Complete DMEM medium with 10% FCS was then added to the Petri dish, in a proportion of 10/1 (v/v), and left overnight at 37°C. The next day the cells were split in 1/20, 1/40 and 1/80 dilutions, and left growing for 17 days after which the foci were scored.

This experimental procedure indicates that, using EGF, the ras oncogene could be internalized into the cell, brought to the nucleus, could be expressed and code for a funtional protein, thereby transforming NIH/3T3 in a stable fashion. The results obtained show that the EGF-ras complex transforms with a greater efficiency than transfections using the calcium phosphate technique. About 5 times more foci were obtained in the 1/40 cell dilution using the EGF-ras complex (Figure 4).

EGF-ras	438 foci
$CaCl_2$, ras	82 foci
Control	8 foci

Foci were gathered, expanded to cultures of $5\text{-}10^6$ cells and injected into athymic nude mice. The EGF-ras colonies as

well as the $CaCl_2$ transfected ras gene form tumors within 3 weeks, whereas control NIH/3T3 cells untreated or just treated with an excess of EGF for 24 hours do not form any tumors in nude mice.

Evidently the invention is not limited to the above embodiments but it can be used in many other applications involving internalization of DNA in-vitro and in-vivo.

Among such applications, the following may be cited:

1. Industrial production of proteins of interest by the inducible or continuous expression of genes efficiently internalized via cytospecific gene vectors according to the present invention. Therefore, large quantities of a protein of intrest can be produced by cells grown in fermentors or by tumor cells carried by laboratory animals. This invention allows one to bypass the long and troublesome steps of establishing cell lines producing a selected protein. It is also possible to internalize RNA complexes which can be directly translated to proteins.

2. Cytospecific drugs consisting of an internalizable molecule, linked to a nucleic acid expressing a protein which can have various types of activities, for example therapeutic, prophilactic, antiviral, cytotoxic, etc... may have an effect on the regulatory mechanism of the cell. The gene can also complement a genetic defect or serve as a market. Such drugs can be used in-vivo for human or animal treatment or in-vitro.

3. The present method of introducing DNA or RNA in the cell gives a whole new dimension to gene therapy because of the possibility of working without using any toxic agents and because it allows efficient targeting of nucleic acids to specific cell populations. Using this internalization technique, modulation of gene expression (e.g. repression) using anti-sense RNA or DNA becomes a very powerful and versatile method.

EXAMPLE 6

A growth factor linked to an anti-sense oligonucleotide inhibits its receptor synthesis.

This is an example where a synthetic oligonucleotide, with a sequence complementary to the epidermal growth factor (EGF) receptor RNA, has been attached to EGF and, after incubation with receptor positive A431 cells, has been able to decrease the EGF receptor synthesis. The direct effect of this was that, due to the lower amount of receptors, the attachment of fresh EGF was inhibited. This new compound has a similar final effect as receptor antagonists since binding of the ligand to the cell is impaired.

We have used in this example a different chemical coupling protocol to attach the synthetic oligonucleotide to the EGF protein. After the oligonucleotide synthesis, a cyclic phosphinamidite (Applied Biosystem, User Bulletin, issue No 38, 1986) was added as described on page 8 of this document. Glutaraldehyde was then used to link the oligonucleotide to the amino group of EGF.

The synthesized oligonucleotide was a 21 nucleotide sequence, complementary to the 5' sequence of the EGF receptor RNA covering the AVG codon and had the following sequence:

5' GT CGC TAC GCT GGG AGG CCCT 3'

This sequence has been chosen so that, once introduced in the cell, it "sticks" specifically to this RNA, blocking the ribosome and stopping the RNA translation which inhibits the EGF receptor synthesis.

The PAGE purified conjugate was then added to trypsinized A431 cells. After different incubation times, the pellets were washed twice and left resting to eliminate the receptor-bound EGF. After an additional wash, $6 \cdot 10^5$cpm ^{125}I-EGF (Amersham) was added and left for incubation with the cells. After two rounds of PBS washes, the cell-bound radioactivity was measured on a gamma counter.

The cells having large quantities of receptors bind large quantities of ^{125}-I-EGF whereas, in contrast, cells having low quantities of receptors bind low quantities of ^{125}I-EGF. The results of this experiment show that when the cells have been submitted to the conjugate, the binding of EGF has been reduced up to 6·6 times as compared to control cells where no conjugate was added.

The receptor concentration was reduced after the cells have been in contact with the EGF conjugate. Therefore, in this example, most surprisingly, after cell internalization, the oligonucleotides have been able to escape from the lysosomal inclusions and have been able to hybridize to the cytoplasmic or nuclear RNA, blocking the receptor synthesis.

This is an example in which targeted anti-sense oligonucleotides can inhibit the synthesis of a specific protein. It is also another example of chemical attachment between the HT (homing/targeting) factor and a nucleic acid which does not degrade their biological activity.

Of course, EGF can be another HT factor and the oligonucleotide can have a different size and composition. Naturally, repeating this example with a linker carrying several oligonucleotides for one HT factor improves the efficiency of the conjugate.

E X A M P L E 7

In-vivo targeting, internalization and expression of a conjugate (HT)-B-(NT)

A plasmid was designed and synthesized with thee following linear structure:

EcoRI BglI BglI HI pvuII EcoRI

HSp70p.	CAT	SV40t	neo	SV40p	PBR322 amp
human HSp70 promotor	chloramphenicol acetyl. transferase gene	SV40 termination signal	neomycin resistance gene	SV40 promotor	PBR322 sequences including ampicillin resistance gene

The linearized plasmid fraction certaining HSp70p-CAT-SV40t was chemically attached to EGF using the imidazole activation protocol described on page 7 and 37 of this document. This conjugate was then injected directly beside A431 tumor xenographs growing in nude mice. After 24 hours, to examine precisely the gene expression, the tumor was removed from the animal and submitted to a heat-shock (43°C, 3 hours). The next day the acetylated chloramphenicol was measured according to standard procedures (Gorman et al.). We could observe a faint acetylated chloramphenicol signal. Although the amount of acetylated chloramphenicol was low, it indicates that some genes had penetrated in the A431 cells and could code for a functional protein.

This example shows that in-vivo targeting and internalization is possible and that, using our technology, injecting (HT)-B-(NT) conjugates in-vivo is a good approach for designing new therapeutic and prevention drugs.

C L A I M S

1. Method for modifying the metabolism of receptor host-cells by introducing therein one or more selected pieces of foreign nucleic acids such as oligo-or polynucleotides, DNA or RNA capable of performing a biochemical function in said host-cells and/or altering the operation of the cell machinery, which comprises the steps of:

(1) making a conjugate adduct by chemically linking said piece(s) of nucleic acid to a ligand promoting or allowing internalization in said cells, said linking being effected by means of a linker or bond which does not impede or repress the biofunctioning of the internalized nucleic acids;

(2) bringing said host-cells into contact with said adduct resulting from step (1), for a time sufficient to achieve effective penetration into the cells and internalization of said linked nucleic acids;

(3) allowing or inducing said foreign nucleic acids to perform said biochemical function in said host-cells, whereby said modification occurs.

2. The method of claim 1, wherein the oligo- or polynucleotides are chosen from RNA, anti-sense RNA, single stranded DNA, duplex DNA, and derivatives thereof, the function of these polynucleotides being to inhibit or modify the cell operation or development.

3. The method of claims 1 or 2 wherein the polynucleotide is a sequence containing at least one functional gene of interest which, in step (3), is expressed in said host-cells.

4. The method of claim 3, wherein the gene codes for an identifiable product and is expressed under control of an untranslated sequence of said polynucleotide.

5. The method of claim 3, wherein the gene codes for RNA which is anti-sense to viral RNA or cellular RNA and performs in the cell to block corresponding viral or cellular functions.

6. The method of claim 3, wherein the gene codes for a biologically active product selected from cytotoxins, hor-

mones, enzymes, antioncogenes, antimitotic agents, anti metabolic agent, lectins, antibodies, proteins of commercial value, etc, under the driving action of an inducible or non-inducible constitutive promotor.

7. The method of claim 6, wherein the promotor is a heat-shock control sequence of eukaryotic origin.

8. The method of claim 1, wherein internalization of said nucleic acids results into transfection or transformation of said host-cells.

9. The method of claim 1, wherein the ligand has high affinity for some receptors of said host-cells and acts as a targeting vector toward said cells.

10. The method of claim 9, wherein said cell receptor is selected among receptors to cell growth factors (e.g. EGF), synthetic and natural derivatives thereof, antibodies and cell specific markers.

11. The method of claim 1, wherein the ligand is selected from EGF, platelet-derived growth factors, α-2-macroglobulin, thrombine, viral-antigens, interleukin, fibroblast growth factor, nerve growth factor, etc.

12. The method of claim 1, wherein said linker is an oligo or polypeptide.

13. The method of claim 12, wherein said polypeptide is a polyamino-acid comprising a number of sequences of foreign nucleic acids bound thereto covalently or by complexation, this number exceeding 10.

14. The method of claim 1, wherein said polypeptide is polyglutamic or polyaspartic acid and the several foreign polynucleotide sequences are bound thereto via the side carboxylate functions thereof.

15. The method of claim 1, wherein the linker is a phosphate diester bond.

16. The method of claim 1, wherein the linker comprises a P-N, N-C, S-S or O-C bond cleavable by endocellular enzymes the breakage of which will liberate the bound internalized polynucleotides.

17. The method of claim 3, comprising the additional step of:

(4) isolating said products of expression of said gene of interest.

18. The method of claim 2, wherein the polynucleotide comprises an inducible gene coding for a product of interest and the host-cell is eukaryotic with unrestricted multiplications, the method comprising the following additional steps of:

(4) inoculating the transformed cell lines into immunodeficient warm-blooded animals which develop tumors in response to inoculation and allowing said tumors to grow to 10^6-10^{10} cells;

(5) dissociating said tumors into individual cells, cultivating said dissociated cells in culture media;

(6) subjecting said culture to stress whereby said gene is expressed; and

(7) collecting and purifying said product of interest.

19. The method of claims 6 and 9, in which the gene codes for a cytotoxin, comprising effecting step (2) by injecting said adduct into the blood stream or a living tissue containing normal cells and tumoral cells bearing said receptor sites, whereby said adduct is selectively internalized in the tumoral cells, then

(3) stressing locally said tissue to induce the production of a cytotoxin or a protein with antitumoral effect, whereby the tumoral cells are inhibited or destroyed while leaving the normal cells unharmed.

20. The method of claim 2 involving operating with at least two independent identical or different polynucleotides, said nucleotides being coupled to one or to two different ligands.

21. As a new composition of matter, a cell transfecting and/or transforming vector consisting of one or more nucleic acid sequences chemically coupled to a ligand capable of internalizing said sequence into host-cells and bound thereto in a manner sufficiently labile in the cell not to repress subsequent biochemical functioning in the cell after internalization.

22. A method of treating a cell mis-functioning condition

in a particular region of the body of a patient which comprises:

(a) introducing a nucleic acid-protein conjugate into said region, said nucleic acid comprising a stress-inducible promoter and a gene operably linked to said promoter which codes for an antitumoral or cytotoxic agent, said protein having a specific affinity for receptors of the diseased cells, whereby said conjugate preferentially targets such cells and is preferentially internalized by such cells; and

(b) locally stressing said region of the body in a manner sufficient to induce expression of said cytotoxic agent.

23. The method of claim 22 in which the stress is heat and the promoter is a heat-shock promoter.

24. The method of claim 22 in which the diseased cells are cancerous and posses receptors for EGF and the protein binds to those receptors.

25. The method of claim 22 in which the gene is transcribed into a message RNA, said RNA being "anti-sense" with respect to a messenger RNA native to said diseased cell, said anti-sense RNA thereby inhibiting cell malfunction and thereby acting as a cytotoxic agent.

26. The method of claim 22 in which the cytotoxic agent is a cytotoxic protein or nucleic acid.

27. The method of claim 26 in which the cytotoxin protein is selected from the group consisting of ricin, abrin, diphtheria toxin, and pseudomonas toxin.

28. The vector of claim 21, in which the ligand has specific affinity toward a receptor, marker or antigen site of the cell, this receptor promoting internalization of said nucleic acids in high yield.

29. The vector of claim 21, in which the internalizing ligand is selected from the group consisting of the following compounds: viral antigens; growth hormone factors like epidermal growth factors, nerve growth factors, platelet-derived growth factors, fibroblast growth factors, α- and β-transforming factors, vaccinia sequences, urogastrone, interleukin, melanocyte-stimulating and thyrotropin releasing hormones,

tansferrin, low density lipoprotein, immunoglobulin, part-B of toxins such as ricin, abrin and diphtheria toxin.

30. The vector of claim 21, in which the nucleic acid sequences are selected from hormone and enzyme producing genes, genes coding for anti-sense RNA capable of blocking the overexpression of EGFR, and oncogenes like ras, myc, Src, c-erb-b2 and neu genes, or that of viral genes like HIV, polio-virus, arbovirus, rhobdovirus, adenovirus, rhinovirus, herpes, influenza, papilloma virus.

31. The vector of claim 21, in which the nucleic acid sequences are selected from short stretches of anti-sense RNA capable of blocking the expression of EGFR, oncogenes and viral genes at the transcriptional and/or translational level.

32. The vector of claim 31, in which the nucleic acid sequences and the ligand are coupled by a bond or linker stable in the plasma but cleaved by endocellular enzymes.

33. The vector of claim 32 in which the linker is an oligopeptide comprising amino acids selected from glycine, alanine, phenylalanine, methionine and related amino-acids.

34. The vector of claim 32, in which the linker is a polyglutamic or polyaspartic polypeptide, the gamma-side chains of which carry nucleic acid sequences.

35. The vector of claim 34 in which the polypeptide carries up to 500 or more identical or different nucleic acid sequences including nucleotides or modified nucleotides like AZT.

36. The vector of claim 34 in which the coupling of the nucleic acid sequences and the linker is effected through an amino-link attached beforehand to said sequence in position 5'.

37. The vector of claim 32, in which the linker comprises an oligopeptide capable of mediating the transport of the nucleic acid sequences from the cytoplasm of the receptor cells to the nucleus.

38. A method for treating or preventing missfunction of selected cells by in-vivo applications of conjugates described in claim 1 and claim 2.

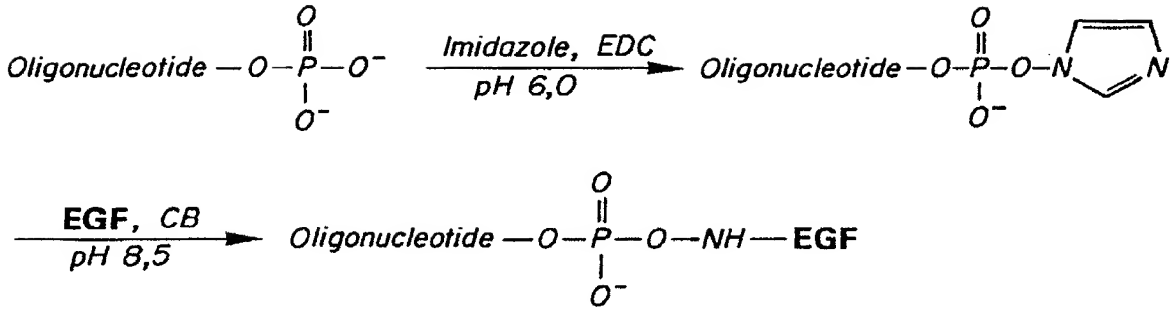

Oligonucleotide —O—P(=O)(O⁻)—O⁻
Imidazole, EDC
pH 6,0
Oligonucleotide —O—P(=O)(O⁻)—O—N
N
EGF, CB
pH 8,5
Oligonucleotide —O—P(=O)(O⁻)—O—NH—EGF

FIG. 1

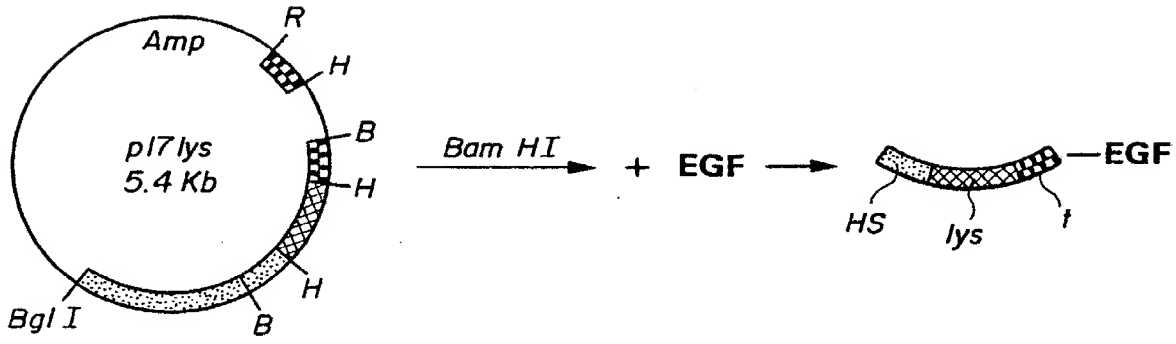

Amp
R
H
B
H
p17 lys
5.4 Kb
H
Bgl I
B
Bam HI
+ EGF
—EGF
HS
lys
t

FIG. 2

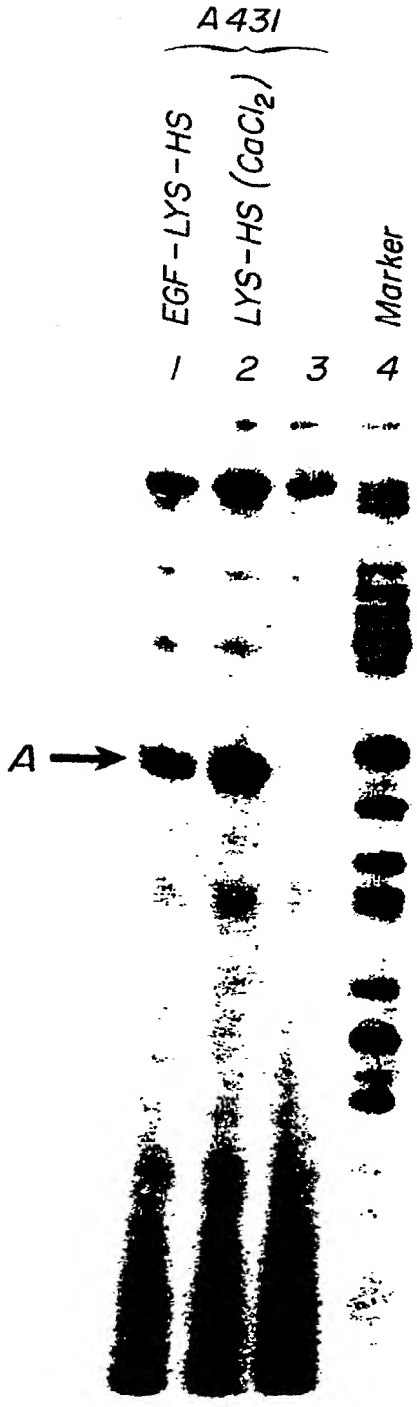


FIG. 3

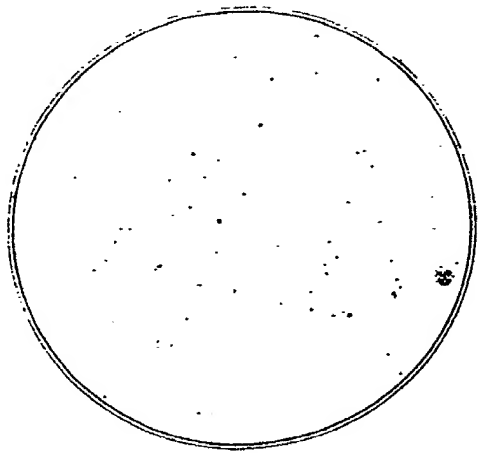


FIG. 4a

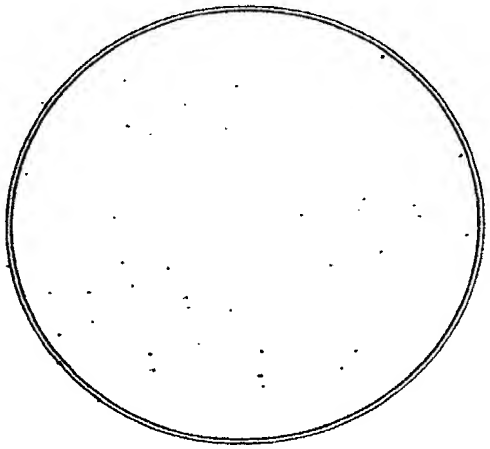


FIG. 4b

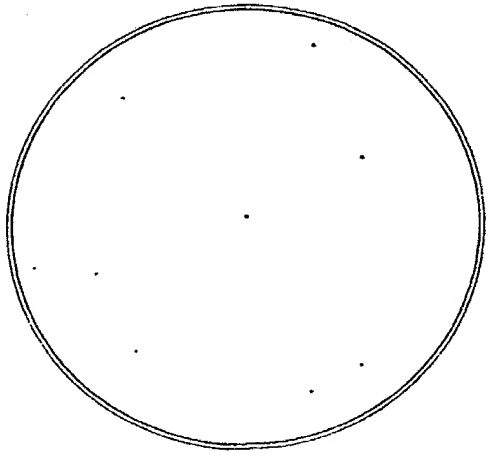


FIG. 4c

FIG. 5a

FIG. 5b

FIG. 6a

FIG. 6b

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 87/00827

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Nucleic Acids Research, volume 11, no. 3, 1983, IRL Press Ltd, (Oxford, GB), S.-Y. Cheng et al.: "A versatile method for the coupling of protein to DNA: synthesis of α_2-macroglobulin-DNA conjugates", pages 654-669 see abstract; discussion	1
Y	Nucleic Acids Research, volume 11, no. 18, 1983, IRL Press Ltd, (Oxford, GB), B.C.F. Chu: "Derivatization of unprotected polynucleotides", pages 6513-6529 see abstract; page 6528, lines 10-21 cited in the application	1
A	Nucleic Acids Research, volume 12, no. 14, 1984, IRL Press Ltd, (Oxford, GB), M.A. Lopata et al.: "High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment"	1

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th April 1988	6 JUN 1988
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	pages 5707-5717 see abstract; introduction; discussion --	
A	Proceedings of the National Academy of Sciences of the USA, volume 76, no. 3, March 1979, (Washington, US), M. Wigler et al.: "DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells", pages 1373-1376 see abstract; page 1373, column 2, line 37 - page 1374, column 2, line 23; table 1; discussion --	1
P,Y	The Journal of Biological Chemistry, volume 262, no. 10, 5 April 1987, The American Society of Biological Chemists, Inc., (US), G.Y. Wu et al.: "Receptor-mediated in Vitro gene transformation by a soluble DNA carrier system", pages 4429-4432 see abstract; introduction; results; discussion --	1
P,Y	Chemical Abstracts, volume 106, no. 23, 8 June 1987, (Columbus, Ohio, US), U. Wienhues et al.: "A novel method for transfection and expression of reconstituted DNA-protein complexes in eukaryotic cells", see page 205, abstract 190339v, & DNA 1987, 6(1), 81-9 --------	1